INFECTION AND IMMUNITY, Dec. 1988, p. 3285–3293
0019-9567/88/123285-09$02.00/0

Vol. 56, No. 12

Virulence Properties and Enterotoxin Production of *Aeromonas* Strains Isolated from Fish

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Received 27 May 1988/Accepted 8 September 1988

The biological activities in vivo and in vitro of 59 motile *Aeromonas* spp. isolated from fish and water tanks were simultaneously analyzed in poikilothermic and homoiothermic systems. A total of 64.3% of the isolates tested were pathogenic for fish, and 62% of *Aeromonas hydrophila* and *A. sobria* isolates either virulent or nonvirulent for fish were enterotoxigenic. Although the majority of the strains were proteolytic and amylolytic and produced DNase, other activities, such as elastase and staphylolysis, were only present in *A. hydrophila*. Most of the strains (96%) produced hemolysins, and 68% had agglutinating capacity, but neither isolates pathogenic for fish nor enterotoxigenic isolates showed specificity for trout or human erythrocytes, respectively. The production of siderophores, agglutination in acriflavine, and precipitation after boiling were found not to be useful tests for screening virulent strains. Although statistical analysis revealed a significant relationship between virulence for fish and positive results for arabinose and sucrose fermentations, elastase, and hemolysis of human erythrocytes, only lysine decarboxylase showed a significant positive relationship with enterotoxigenicity. Using extracellular products from representative *Aeromonas* strains with different virulence markers and belonging to distinct O serogroups, we demonstrated a lack of correlation between cytotoxicity for fish and homoiothermic cell lines and pathogenicity. The extracellular products from selected pathogenic *A. hydrophila* strains were lethal for rainbow trout and displayed proteolytic, hemolytic, and cytotoxic activities which were simultaneously lost after heat treatment. The findings reported here indicate that it is not possible to establish a common and single mechanism involved in the invasion of *Aeromonas* spp. in poikilothermic and homoiothermic hosts.

Motile *Aeromonas* spp. are widely distributed in nature and have been recognized as normal microbial flora of aquatic and terrestrial organisms (17, 42) as well as opportunistic pathogens for fish and other poikilothermic and homoiothermic animals (4, 9, 13, 29, 39). Several biochemical properties and virulence factors (i.e., proteases, hemolysins, enterotoxins, cytotoxins, dermonecrotic factor) have been reported as potential indicators of pathogenicity in *Aeromonas hydrophila*, but their roles in fish and human diseases are not clearly determined (2, 6, 14, 19, 26, 33, 43). In addition, the number and nature of toxic factors in extracellular products (ECP) of important fish pathogens such as *Aeromonas* and *Vibrio* species are not yet elucidated. Whereas some authors have reported that the toxic fraction of the ECP is associated with hemolytic activity (2, 27), other workers consider that proteases are the main virulence factors implicated in the pathological effects produced by culture filtrates (12, 16, 18, 31, 34, 35). On the other hand, Lallier et al. (19) concluded that an undefined dermonecrotic factor present in the ECP of *A. hydrophila* strains was responsible for fish mortality.

Until the present, the majority of studies of motile *Aeromonas* spp. analyzed separately the mechanism of pathogenicity only for fish (2, 14, 19, 34, 44) or for humans (6, 8, 17, 26, 43). To our knowledge, this study represents the first report in which the biological activities of *Aeromonas* isolates are evaluated simultaneously in terms of their effects on mammal and fish cell systems. In addition, the toxicity in vivo and in vitro of the ECP of selected strains was investigated. The possible relationship among phenotypic characteristics, the ability to produce certain virulence factors, and animal pathogenicity was also determined.

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MATERIALS AND METHODS

Bacterial strains and biochemical characterization. We used 49 motile *Aeromonas* spp. isolated from different organs of moribund and apparently healthy rainbow trout (*Salmo gairdneri*) as well as from the water in fish holding tanks. The following 10 reference strains from the American Type Culture Collection, Rockville, Md., and private donors were included in all assays: ATCC 7966, ATCC 15467, 1.25, 1.54, Y-62, 67-P-24, 81-83, 80-A1, 80-A2, and 80-A3. As previously described (40), 39 strains were *A. hydrophila*, 3 were identified as *A. caviae*, and 14 were assigned to the species *A. sobria*. Three strains could not be differentiated beyond the genus level (see Table 1).

In the enterotoxin and cytotoxin assays, clinical isolates of *A. hydrophila* (6043 and 6075) and *A. sobria* (3700 and 3385) were also tested. These four strains were supplied by V. Burke, Princess Margaret Children's Medical Research Foundation, Perth, Australia. All strains were routinely cultured on tryptic soy agar (TSA) or in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 25°C for 24 to 48 h, stored on TSA slants at 4°C under mineral oil, and frozen at −70°C with 15% (vol/vol) glycerol. In general, the assays were performed simultaneously with cultures grown on TSA and blood agar (TSA with 5% defibrinated sheep blood) plates.

Serological testing. Slide agglutination tests were conducted to examine the serological relationships of selected *Aeromonas* spp. as previously described (38). Rabbit antisera were raised from *A. hydrophila* B-32, *A. hydrophila* B-51, and *A. caviae* ATCC 15467. The reactions were

conducted by using, for each strain, the whole cells and the heat-stable somatic O antigens which were obtained by heating the respective cell suspensions (10% [vol/vol] in phosphate-buffered saline [PBS]) at 100°C for 1 h.

Virulence for fish. The pathogenicity of *Aeromonas* isolates was determined by intraperitoneal inoculation of fingerling rainbow trout as previously described (37). The assays were carried out at 18 to 20°C with six to eight fish (3 to 4 g) per dose (0.1 ml per fish). The degree of virulence, expressed as the 50% mean lethal dose (LD_{50}), was calculated by the method of Reed and Müench (30).

Enzymatic activities and production of hemolysins. Caseinase, gelatinase, amilase, lipase, phospholipase, and DNase activities and esculin hydrolysis were evaluated by a plate assay method basically as described by West and Colwell (45) and Wakabayashi et al. (44). Elastase activity and staphylolysis were determined as described by Hsu et al. (14). For each test, 24-h cultures of the isolates grown in tryptic soy broth were inoculated in the respective substrates with a multipoint inoculator (Denley, Billingshurst, United Kingdom).

To evaluate the specificity of hemolysins for erythrocytes of different species, we used blood from homoiothermic and poikilothermic sources such as guinea pigs, sheep, humans, rainbow trout, and Pacific salmon. Hemolytic activity was detected on TSA plates containing the respective erythrocytes at a 5% (vol/vol) final concentration. Results were recorded after 24 and 48 h of incubation at 15 and 25°C to verify the possible influence of temperature on the hemolytic patterns.

Detection of siderophore activity. The production of diffusible siderophore compounds by the *Aeromonas* strains was tested on blue agar by the method of Schwyn and Neilands (32). The dye chrome azurol S (Sigma Chemical Co., St. Louis, Mo.) incorporated into the medium can form stable complexes with iron. The production of a siderophore (which removes the iron from the complex) is indicated by a change of the color to orange around the colony after 48 h of incubation at 25°C.

The capacity to produce the phenolate-type enterobactin and the hydroxamate-type aerobactin was determined by testing the ability of cellular supernatants from bacterial cultures to cross-feed different mutants defective in the iron uptake system (22). Two mutants of *Salmonella typhimurium* LT-2 deficient in the biosynthesis of enterobactin (strain *enb-1*, which can only use enterobactin, and strain *enb-7*, which can use enterobactin and the intermediate 2,3-dihydroxybenzoic acid) and *Escherichia coli* LG1522, a mutant deficient in aerobactin biosynthesis but possessing an aerobactin receptor, were included in the bioassays as indicator strains. These three organisms were provided by J. H. Crosa, Oregon Health Sciences University, Portland.

Agglutination assays and inhibition of hemagglutination. *Aeromonas* strains grown on TSA and blood agar plates at 15 and 25°C were harvested after 12, 24, and 42 h of incubation in PBS (pH 7.4) and adjusted to yield 5×10^{10} cells per ml. Human group O blood obtained by venipuncture and rainbow trout blood collected by cardiac puncture were immediately placed into Alsever solution (GIBCO Laboratories, Grand Island, N.Y.) and stored for not more than 1 week at 4°C. For the agglutination tests, erythrocytes and yeast cells (*Saccharomyces cerevisiae*) were washed in PBS, and a 3% (vol/vol) final suspension was prepared in this saline solution.

Agglutination assays were carried out basically as previously described (36). The minimum hemagglutinating dose was defined as the smallest number of bacterial cells per milliliter that produced visible agglutination in 10 min, and the hemagglutination power was calculated as 10^{11} per minimum hemagglutinating dose (11). The hemagglutination inhibition assay was performed essentially the same way as the agglutination assay with bacterial suspensions containing 10 minimum hemagglutinating doses and 1% (wt/vol) solutions in PBS of D-mannose, D-galactose, and L-fucose (Sigma).

Acriflavine test and stability after boiling. Agglutination in acriflavine and stability after boiling were evaluated by the method of Mittal et al. (25). Slide agglutination in acriflavine (0.2%) was conducted with colonies from TSA and blood agar plates incubated for 24 h. Precipitation of the cells was verified after boiling an 18-h-old culture in brain heart infusion broth (Difco) for 1 h.

Enterotoxigenicity assay. The enterotoxin production of *Aeromonas* strains was measured by the suckling mouse test. Filtered supernatants (100 μl) of the respective bacterial cultures were administered into the stomachs of 2- to 4-day-old mice. After incubation for 3 h at 30°C, animals were sacrificed, and the fluid accumulation ratio was calculated as the ratio of intestinal weight to remaining body weight. Ratios above 0.08 were regarded as positive tests. Culture filtrates of some enterotoxin-positive strains were treated at 100°C for 15 min, and the fluid accumulation ratios induced by heated and unheated solutions were determined.

E. coli B2C (heat-labile and heat-stable enterotoxin positive) (supplied by T. M. Gleeson, Bureau of Microbial Hazards, Ottawa, Ontario, Canada), *Vibrio cholerae* LA4808 (cholera enterotoxin positive) (provided by R. R. Colwell, Department of Microbiology, University of Maryland, College Park), and *E. coli* K-12 strain 185 (heat-labile and heat-stable enterotoxin negative) (from our own collection) were used as positive and negative controls.

Extraction of ECP and detection of proteases and hemolysins. The ECP of *Aeromonas* strains were obtained by the cellophane plate technique (23). After 48 h of incubation at 25°C, grown cells were washed off the cellophane with the minimum volume of PBS solution. These cell suspensions were centrifuged at 10,000 × *g* for 15 min, and the supernatants obtained were filtered through 0.45-μm-pore membranes and stored at both 4 and −30°C until needed. The protein concentrations of all ECP were determined by the method of Bradford (5) with Bio-Rad reagent (Bio-Rad Laboratories, Munich, Federal Republic of Germany).

Proteolytic and hemolytic activities of ECP were measured as described by Sakai (31) and Hastings and Ellis (12), respectively, with sodium caseinate for the protease assay and trout and human erythrocytes for the determination of hemolytic capacity. One unit of proteolytic activity was expressed as a change in the A_{280} of 0.01. The hemolytic titer was expressed as the reciprocal of the highest dilution of crude ECP producing complete hemolysis. The stability of hemolysins and proteases was assayed after the samples were heated at 60, 70, 80, and 100°C for 15 min.

Toxic activities of the ECP in in vivo and in vitro assays. Groups of 6 or 10 fingerling rainbow trout were intraperitoneally inoculated with 0.1 ml of each ECP preparation and maintained for 1 week under the conditions described above. The live cells of the respective bacteria were inoculated simultaneously as positive controls.

Filtered supernatants obtained from bacterial cultures in tryptic soy broth and ECP preparations (cellophane plate method) of selected *Aeromonas* strains were assayed for cytotoxicity as previously described (36) in the following fish

and homoiothermic cell lines: CHSE-214 (chinook salmon embryo), RTG-2 (rainbow trout gonad), FHM (fathead minnow peduncle), EPC (epithelioma papillosum of carp), Vero (African green monkey kidney), L-929 (mouse lung fibroblast), and HeLa (human cervix epithelioid carcinoma). Cell monolayers grown in 24-well plates (Costar, Cambridge, Mass.) were inoculated with 0.1 ml of serial twofold dilutions of each sample and incubated at 18°C (fish cell lines) or 37°C (Vero, L-929, and HeLa cell lines). Total or partial destruction of monolayers within a 3-day period was scored as a positive cytotoxic effect. Samples treated at 60 and 80°C for 15 min were also inoculated. The strains of *E. coli* and *V. cholerea* listed above were included as controls.

Statistical analysis. The possible relationships among virulence for fish, enterotoxigenicity, and phenotypic characteristics were assessed with the chi-square test. Probabilities lower than 0.1 ($P < 0.1$) were considered significant. The phenotypic traits analyzed were as follows: Voges-Proskauer, lysine decarboxylase, arabinose and sucrose fermentations, elastase, and hemolytic and hemagglutinating activities of trout and human erythrocytes.

RESULTS

Pathogenicity of the strains for fish and enterotoxin production. The virulence assays demonstrated that of 56 isolates tested, 36 (64.3%) were pathogenic for fingerling rainbow trout. According to the degree of virulence described by Mittal et al. (25), 22 strains were included in the virulent category (LD_{50}, 10^4 to 10^5), 14 strains were classified as weakly virulent (LD_{50}, 10^6 to 10^7), and the remaining *Aeromonas* spp., displaying LD_{50} of $>10^8$, were considered avirulent (Table 1). We found that whereas a great number of *A. hydrophila* strains (28 of 39) were pathogenic, a high proportion (8 of 14) of *A. sobria* strains were nonpathogenic.

The suckling mouse assay revealed that 21 of 34 (62%) fish *Aeromonas* strains assayed produced enterotoxins; there was a similar frequency of enterotoxigenic strains between *A. hydrophila* (70%) and *A. sobria* (60%). Of the three *A. caviae* isolates, only avirulent strain 1.25 was enterotoxigenic. Interestingly, 13 of 21 (61.9%) enterotoxigenic and 9 of 13 (69.2%) nonenterotoxigenic *Aeromonas* strains were virulent for fish. It is noteworthy that one of the four clinical isolates (*A. hydrophila* 6075), included for comparative purposes, was not enterotoxigenic, displaying the lowest fluid accumulation ratio (0.054). Treatment at 100°C for 15 min of culture filtrates of some positive strains revealed that the enterotoxins of motile *Aeromonas* strains varied in their stability against heat.

Production of extracellular enzymes, hemolysins, and siderophores. The majority (more than 96%) of the 59 *Aeromonas* strains tested were proteolytic and amylolytic and produced DNase. Regardless of the species considered, a large number of isolates also produced lipase (81.3%) and phospholipase (69.4%) and hydrolyzed esculin (76.2%). However, the lowest percentage (42.4%) was detected for elastase activity, which was present only in strains of *A. hydrophila* and always in association with staphylolysis (Table 1).

The hemolytic assays indicated that nearly all (57 of 59) of the strains displayed some lytic activity (Table 1). However, if we consider the different sources of erythrocytes tested, three predominant patterns were found: two patterns (including 39 strains) showed a wide spectrum of hemolysis of homoiothermic and poikilothermic erythrocytes, and one pattern (16 strains) showed specificity for poikilothermic erythrocytes. Interestingly, neither strains pathogenic for fish nor enterotoxigenic strains showed specificity for fish or mammal erythrocytes, respectively. The temperature of incubation (15 or 25°C) did not affect the pattern of hemolysis.

When the chemical method of Schwyn and Neilands (32) was used to determine the presence of siderophore activity, all strains were positive (Table 1). However, some strains, regardless of their pathogenic properties, displayed stronger positive responses than did others, which showed a reduced orange halo on blue agar plates. The bioassays conducted to detect some specific hydroxamate or phenolate siderophores in a group of selected *Aeromonas* strains (see Table 2) indicated that these isolates failed to produce both enterobactin and aerobactin.

Hemagglutinating activity and sugar inhibition patterns. The capacity to adhere to host cells was evaluated by agglutination assays with human and trout erythrocytes and *S. cerevisiae* cells. Of 55 strains assayed, 38 (68%) had agglutinating capacity. Three distinct patterns were established among the *Aeromonas* spp.: one pattern (19 strains) showed a broad spectrum of adhesins for both types of erythrocytes and yeast cells, a second pattern (6 strains) showed activity for human erythrocytes and yeast cells, and a third pattern (13 strains) showed agglutination of only yeast cells (data not shown). A lack of specific hemagglutination of trout or human erythrocytes was detected, respectively, in strains pathogenic for fish or enterotoxigenic isolates (Table 1). In addition, 11 highly virulent and 8 weakly virulent *Aeromonas* isolates were negative for hemagglutination.

The agglutination reactions of the 25 hemagglutination-positive strains were quantitated, and the hemagglutination power ranged between 10^2 and 10^4, only 2 strains (*A. hydrophila* 1.54 and *A. sobria* P-355) displayed a hemagglutination power higher than 10^4 (data not shown). The sugar inhibition studies showed that, in general, hemagglutination was sensitive to inhibition by D-mannose, usually in association with L-fucose. Only three virulent and nonenterotoxigenic isolates (1.54, 67-P-24, and P-355) displayed no inhibition of hemagglutination with any of the sugars used. In addition, whereas some strains showed the same sugar inhibition pattern regardless of the source of erythrocytes, other strains showed different patterns according to the erythrocyte source. The adhesin expression in vitro of the *Aeromonas* spp. was not influenced by the growth medium used, the temperature, or the length of incubation.

Additional tests related to cell surface characteristics. Agglutination in acriflavine (Acr) and precipitation after boiling (PAB) were evaluated in our *Aeromonas* isolates (Table 1). The results indicated that 20 of 34 (58.8%) strains pathogenic for fish were Acr^- and that 8 of 30 (26.7%) were PAB^+. Similar percentages for both characteristics were observed with the avirulent strains. On the other hand, the Acr^- phenotype seemed to be associated more with enterotoxigenicity, since 14 of 20 (70%) enterotoxigenic strains and only 4 of 12 (33.3%) nonenterotoxigenic strains were Acr^-. However, the PAB^+ property was only present in 5 of 18 (27.7%) enterotoxigenic strains. Therefore, only five *Aeromonas* spp. (three of them virulent for fish) simultaneously possessed the Acr^- PAB^+ phenotype.

Relationships among virulence for fish, enterotoxigenicity, and phenotypic characteristics. Although Table 1 indicates that none of the phenotypic traits analyzed can be used as an absolute indication of pathogenicity, the possible relationship between several putative virulence markers (biochemical properties, hemolysin production, and hemagglutinating activity) and pathogenicity for fish or enterotoxigenicity was

TABLE 1. Different virulence characteristics of the motile *Aeromonas* strains used in this study[a]

Strain	Species	LD_{50}[b]	Enterotoxigenicity[c]	Elastase/staphylolysis	Hemolysis against erythrocytes from:		Hemagglutination of erythrocytes from:		Acr[d]	PAB	Siderophore production[e]
					Humans	Trout	Humans	Trout			
Highly virulent											
80-A1	*A. hydrophila*	6.4×10^4	+ (0.085)	+/+	+	+	−	−	+	+	+
T-80	*A. hydrophila*	2×10^4	+ (0.13)	+/+	+	+	−	−	−	−	(+)
B-36	*A. hydrophila*	5.7×10^4	+ (0.12)	+/+	+	+	−	−	−	+	(+)
T-12	*A. hydrophila*	4×10^5	+ (0.11)	+/+	+	+	−	−	+	−	ND
B-51	*A. hydrophila*	1.4×10^5	+ (0.082)	+/+	+	+	+	+	−	−	(+)
B-37	*A. hydrophila*	8×10^4	+ (0.095)	+/+	+	+	(+)	(+)	−	ND	(+)
T-2	*A. hydrophila*	5×10^5	+ (0.10)	−/−	+	+	−	−	+	−	+
P-192	*A. hydrophila*	3.6×10^5	+ (0.10)	−/−	−	+	−	−	−	−	+
P-261	*A. sobria*	1.4×10^5	+ (0.11)	−/−	+	+	+	+	−	−	ND
P-33	*A. sobria*	9×10^4	+ (0.090)	−/−	−	+	ND	ND	ND	ND	+
B-32	*A. hydrophila*	3.2×10^4	− (0.070)	+/+	+	+	−	−	−	−	(+)
T-16	*A. hydrophila*	4×10^4	− (0.072)	+/+	+	+	(+)	−	+	−	(+)
1.54	*A. hydrophila*	2×10^5	− (0.068)	+/+	+	+	+	+	(+)	+	+
B-31	*A. hydrophila*	9×10^4	− (0.075)	+/+	+	+	+	+	−	+	(+)
67-P-24	*A. hydrophila*	8.6×10^4	− (0.077)	+/+	+	+	+	+	+	−	+
P-34	*A. sobria*	6.4×10^5	− (0.074)	−/−	−	+	ND	ND	ND	ND	+
80-A3	*A. hydrophila*	8.2×10^4	ND	+/+	+	+	−	−	+	+	(+)
B-35	*A. hydrophila*	6.5×10^4	ND	+/+	+	+	+	+	−	+	(+)
F-13	*A. hydrophila*	7.4×10^5	ND	+/+	+	+	+	+	−	−	+
P-153	*A. hydrophila*	4.8×10^5	ND	−/−	+	+	−	−	−	−	+
P-172	*A. hydrophila*	5.6×10^5	ND	−/−	+	+	−	−	−	−	+
F-3	*A. hydrophila*	5.2×10^5	ND	−/−	−	+	−	−	−	ND	+
Weakly virulent											
A-12	*A. hydrophila*	2.7×10^7	+ (0.083)	+/+	+	+	−	−	−	−	+
B-11	*A. hydrophila*	8.5×10^6	+ (0.091)	+/+	+	+	−	−	−	−	(+)
A-7	*A. sobria*	7×10^6	+ (0.10)	−/−	−	+	ND	ND	−	−	+
P-355	*A. sobria*	6.2×10^6	− (0.070)	−/−	+	+	+	+	(+)	−	+
ATCC 15467	*A. caviae*	6.8×10^7	− (0.074)	−/−	−	+	+	+	+	+	+
P-322	*A. caviae*	5.2×10^6	− (0.074)	−/−	−	+	−	−	+	−	+
P-212	*A. hydrophila*	4.3×10^6	ND	+/+	+	+	−	−	+	−	(+)
A-10	*A. hydrophila*	1.3×10^7	ND	+/+	+	+	−	−	−	−	+
A-11	*A. hydrophila*	9×10^6	ND	+/+	+	+	−	−	−	−	ND
C-61	*A. hydrophila*	5.4×10^7	ND	+/+	+	+	−	−	−	ND	+
P-142	*A. hydrophila*	6.7×10^6	ND	−/−	+	+	+	+	+	−	+
ATCC 7966	*A. hydrophila*	3.5×10^6	ND	−/−	+	+	+	−	+	+	+
P-54	*A. hydrophila*	2×10^6	ND	−/−	+	+	−	−	+	ND	+
A-9	*A. sobria*	2.5×10^7	ND	−/−	+	+	+	+	−	−	+
Avirulent											
P-226	*A. hydrophila*	$>10^8$	+ (0.092)	+/+	+	+	−	−	(+)	−	(+)
81-83	*A. hydrophila*	$>10^8$	+ (0.082)	+/+	+	+	−	−	−	−	+
P-74	*A. hydrophila*	$>10^8$	+ (0.11)	−/−	−	+	−	−	−	+	ND
C-81	*A. hydrophila*	$>10^8$	+ (0.10)	−/−	−	+	−	−	−	ND	+
P-252	*A. sobria*	$>10^8$	+ (0.10)	−/−	−	+	+	+	−	−	+
P-337	*A. sobria*	$>10^8$	+ (0.11)	−/−	+	+	+	+	−	+	ND
1.25	*A. caviae*	$>10^8$	+ (0.13)	−/−	+	−	+	+	+	+	+
P-241	*A. sobria*	$>10^8$	+ (0.11)	−/−	−	+	+	+	+	−	ND
80-A2	*A. hydrophila*	$>10^8$	− (0.072)	+/+	+	+	+	−	−	−	(+)
A-2	*A. sobria*	$>10^8$	− (0.075)	−/−	+	+	+	+	−	−	+
P-281	*A. sobria*	$>10^8$	− (0.055)	−/−	−	+	−	−	+	−	+
Y-62	*A. hydrophila*	$>10^8$	ND	+/+	+	+	+	−	+	+	+
A-4	*A. hydrophila*	$>10^8$	ND	+/+	+	+	+	−	+	−	+
P-254	*A. hydrophila*	$>10^8$	ND	−/−	+	−	+	−	−	−	+
P-202	*A. hydrophila*	$>10^8$	ND	−/−	−	+	−	−	(+)	−	+
P-232	*A. hydrophila*	$>10^8$	ND	−/−	−	+	−	−	−	−	+
C-82	*A. hydrophila*	$>10^8$	ND	−/−	−	+	−	−	−	ND	+
P-334	*A. sobria*	$>10^8$	ND	−/−	+	+	−	−	(+)	+	+
P-351	*A. sobria*	$>10^8$	ND	−/−	−	+	ND	ND	ND	−	+
P-272	*A. sobria*	$>10^8$	ND	−/−	+	+	+	+	−	−	+
F-9	*Aeromonas* sp.	ND	− (0.075)	−/−	−	−	−	−	+	−	ND
B-38	*Aeromonas* sp.	ND	ND	−/−	−	−	−	−	−	−	+
C-71	*Aeromonas* sp.	ND	ND	−/−	+	+	+	+	−	−	+

[a] +, Positive response; −, negative response; (+), weak and delayed positive response; ND, not determined.
[b] Number of viable cells needed to kill 50% of inoculated fish in a 7-day period.
[c] Determined by the suckling mouse assay. Data in parentheses express the fluid accumulation ratio. Ratios above 0.08 were considered positive.
[d] Acriflavine was used at a concentration of 0.2%.
[e] Tested by the method of Schwyn and Neilands (32).

TABLE 2. Serological reactions and cytotoxic activities of a selected group of motile *Aeromonas* strains[a]

Strain	Virulence for fish/ enterotoxigenicity	O antigen agglutination with antisera from:			Cytotoxic response on:						
					Fish cell line[b]				Homoiothermic cell line[c]		
		B-51	B-32	ATCC 15467	RTG-2	CHSE-214	FHM	EPC	HeLa	Vero	L-929
Fish isolates											
A. hydrophila											
B-51	+/+	+	(+)	−	+	+	+	+	+	+	+
80-A1	+/+	(+)	(+)	(+)	+	+	+	+	+	+	+
B-36	+/+	+	+	+	+	+	+	+	ND	ND	ND
T-12	+/+	−	−	−	+	+	+	+	+	+	+
T-2	+/+	+	+	+	+	+	+	+	+	+	+
B-32	+/−	+	+	+	+	+	+	+	+	+	+
B-35	+/ND	+	+	+	+	+	+	+	+	+	+
P-226	−/+	(+)	(+)	(+)	+	+	+	+	+	+	+
80-A2	−/−	−	−	−	+	+	+	+	+	ND	+
A. sobria											
P-261	+/+	−	−	−	+	+	+	+	+	+	+
P-33	+/+	(+)	(+)	+	−	−	−	−	−	−	−
A-2	−/−	−	−	−	+	+	+	+	+	+	+
P-281	−/−	+	(+)	−	−	−	−	−	−	−	−
A. caviae											
ATCC 15467	+/−	(+)	(+)	+	−	−	−	−	−	−	−
P-322	+/−	−	−	+	−	−	−	−	−	−	−
Clinical isolates											
A. hydrophila											
6075	−/−	−	−	−	+	+	+	+	+	+	+
6043	ND/+	−	−	−	+	+	+	+	+	+	+
A. sobria											
3700	−/+	(+)	(+)	(+)	+	+	+	+	+	+	+
3385	ND/+	−	−	−	+	+	+	+	+	+	+
Controls											
E. coli B2C	ND/+				−	−	−	−	−	+	−
E. coli K-12 strain 185	ND/−				−	−	−	−	−	−	−
V. cholerae LA4808	ND/+				(+)	(+)	+	+	(+)	+	+

[a] See Table 1, footnote *a*.
[b] Toxic effect recorded after 6 h at 18°C.
[c] Toxic effect recorded after 6 h at 37°C.

evaluated by the chi-square test. A significant relationship ($P < 0.1$) was found between virulence for fish and positive results for each of the following characteristics: production of acid from arabinose and sucrose, elastase, and hemolytic activity for human erythrocytes.

In the group of 34 strains randomly chosen for the suckling mouse assay, we found that only the lysine decarboxylase test showed a highly significant positive relationship ($P < 0.05$) with enterotoxin production. No association was detected between virulence for fish and enterotoxigenic capacity.

Cytotoxic activity of ECP. Fifteen representative fish *Aeromonas* strains with different virulence markers and belonging to distinct O serogroups were selected to evaluate their cytotoxic effects in in vitro assays (Table 2). The tests showed that the ECP obtained from our pathogenic and nonpathogenic *A. hydrophila* strains displayed positive responses in all of the fish and homoiotherm cell lines tested within 3 or 6 h. Only two *A. sobria* strains and one *A. caviae* strain failed to produce cytotoxic effects. The minimal dose necessary to produce partial or total monolayer destruction ranged from 10 to 20 units of proteolytic activity. Although some differences were observed depending on the cell line and ECP sample used, in general degenerative changes were manifested by pyknotic nuclei, vacuolization, rounding, shrinking, dendritic elongation, and final cell detachment (Fig. 1). Interestingly, enterotoxigenic and nonenterotoxigenic clinical isolates of *Aeromonas* spp. showed a cytotoxicity pattern similar to that exhibited by the fish isolates. Heat treatment of positive samples at 60°C resulted in a decrease in cytotoxic activity, which was totally lost after treatment at 80°C for 15 min.

Toxicity of the ECP for fish. We selected three *A. hydrophila* strains pathogenic for fish to evaluate the toxic effects of their ECP in rainbow trout. One nonpathogenic isolate was also included for comparative purposes. Fish mortality occurred 24 to 48 h after intraperitoneal inoculation of the respective ECP. The lethal dose ranged from 18 to 35 μg of protein per g of fish (Table 3), and hemorrhage and necrosis at the injection site were the common signs observed in all

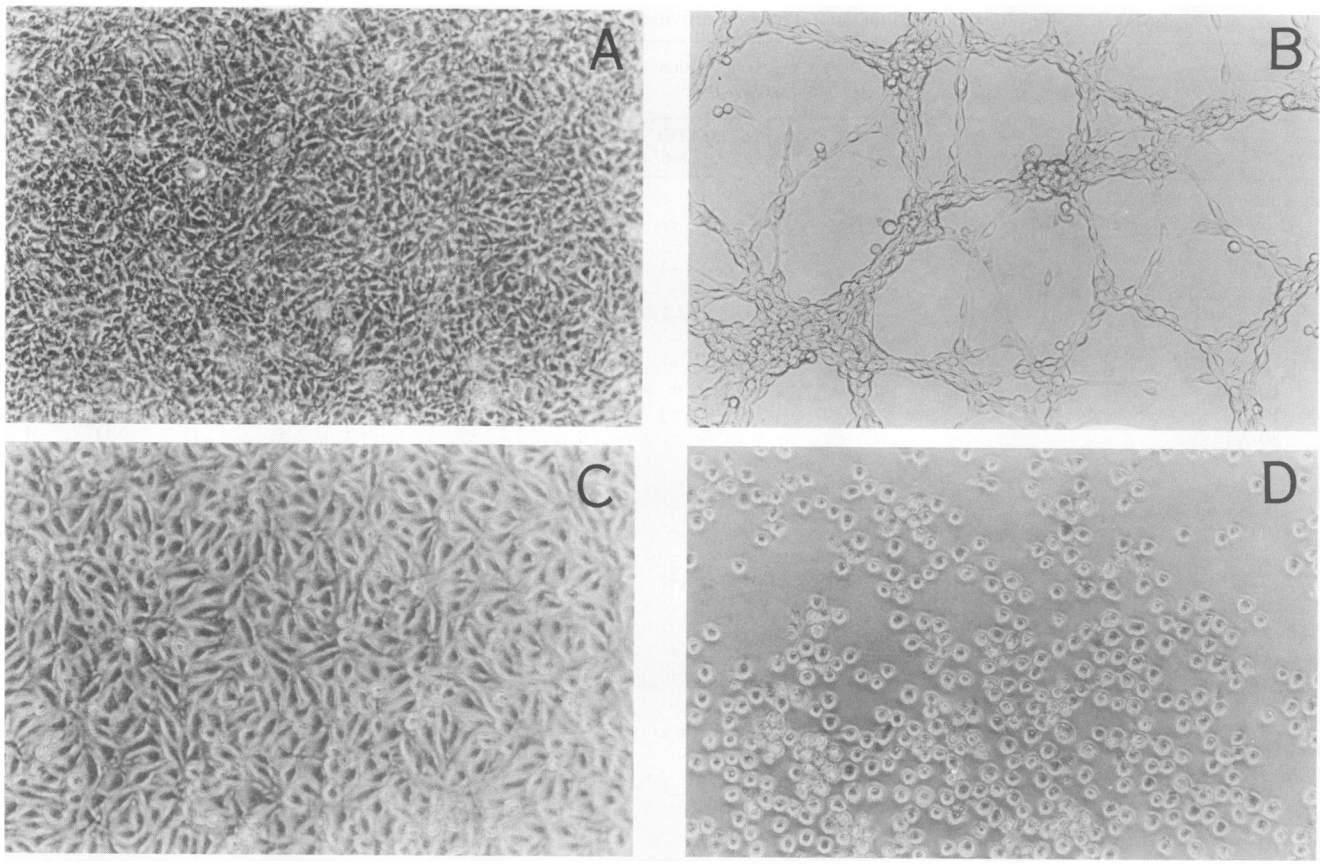

FIG. 1. Cytotoxic effects of the ECP of *A. hydrophila* B-32 on the EPC fish cell line and on the mouse L-929 cell line. (A) Control EPC cells. (B) Initial cytotoxic response on EPC cells manifested by clusters of rounding cells, cell shrinkage, and dendritic elongation. (C) Control L-929 cells. (D) Final toxic effects on L-929 cells manifested by total rounding and cell detachment.

dead fish. The ECP were more effective than the respective live cells (shorter time to death). As we expected, the ECP obtained from nonvirulent *A. hydrophila* P-226 did not have any visible toxic effect, although, interestingly, they had the most notable proteolytic activity.

Whereas heat treatment of the ECP samples revealed that caseinase and hemolytic activity against trout erythrocytes were gradually reduced and thoroughly lost after heating at 80°C for 15 min, hemolytic activity against human erythrocytes was totally lost at 60°C. Pathological effects were not observed in fish inoculated with inactivated fractions (80°C for 15 min) of the different ECP preparations.

DISCUSSION

Motile *Aeromonas* spp. show a wide variation in virulence but, at present, it is not clear whether *A. hydrophila* and *A. sobria* have more importance as fish or human pathogens (4,

TABLE 3. Comparison of lethal toxicity and proteolytic and hemolytic activities of ECP in four *A. hydrophila* isolates

Strain	Initial cell suspension concn (mg/ml)	Total protein (mg/ml)	Proteolytic activity (U/mg of protein)[a]	Hemolytic activity (HU/mg of protein) in[b]:		Fish mortality (no. dead/ no. tested)[c]	Mean time to death (h)	Lethal dose (μg of protein/g of fish)
				Trout	Humans			
Virulent								
B-51	459	1.05	1,027	1,625	203	5/6	97	35.0
B-32	230	0.55	2,000	1,800	ND	5/5	40	18.3
80-A1	264	0.76	1,129	1,122	560	3/5	57	25.3
Avirulent (P-226)	210	0.50	4,685	ND[d]	ND	0/4		

[a] One proteolytic unit (U) was defined as a change in the A_{280} of 0.01.
[b] One hemolytic unit (HU) was expressed as the reciprocal of the highest dilution of ECP producing complete hemolysis.
[c] Rainbow trout were inoculated intraperitoneally with 0.1 ml of each ECP.
[d] ND, Not determined.

6, 14, 19, 25). Although they can produce extracellular toxins or enzymes, until the present report the biological activities of these ECP were not simultaneously evaluated in poikilothermic and homoiotherm cells and systems.

In agreement with several authors (14, 33, 44), we found a positive relationship between virulence for fish and the presence of elastase and staphylolytic enzymes. However, the fact that in our study these activities were observed in isolates of *A. hydrophila* but not in the other *Aeromonas* species (Table 1) indicates that these traits are not a differential index for defining potential virulent strains. We consider, as do Wakabayashi et al. (44), that these enzymatic activities are valuable taxonomic criteria for the identification of *A. hydrophila*.

A relationship between hemolysin production and fish hemorrhagic septicemia as well as human illness caused by motile *Aeromonas* spp. has been reported by some authors (2, 43). In this work the majority of strains (96%) were hemolytic regardless of their virulence for fish or enterotoxigenic capacity (Table 1). Therefore, hemolysins can be one of several factors determining pathogenicity but are not required for virulence in all *Aeromonas* species. In addition, the different hemolysis patterns exhibited (depending of the source of erythrocytes) and the results of heat treatment of ECP samples from selected *Aeromonas* strains seem to indicate the production of distinct hemolysins against poikilothermic and homoiothermic erythrocytes. Similarly, siderophore activity was detected in all the strains (Table 1), but the positive responses differed, possibly indicating the production of siderophores with distinct iron affinities. We have reported analogous findings with *V. anguillarum* strains (22). Although it is known that bacteria can produce some siderophore compounds simply for survival, the cross-feeding assays demonstrated that at least aerobactin and enterobactin biosyntheses are not essential virulence determinants in our motile *Aeromonas* strains.

The agglutination assays conducted to determine the adherence of our *Aeromonas* strains to host cell surfaces revealed that some pathogenic strains were agglutination negative. In addition, neither isolates virulent for fish nor enterotoxigenic isolates specifically hemagglutinated trout or human erythrocytes, respectively. A similar lack of specificity was observed in clinical *Aeromonas* spp. (26) and in pathogenic and environmental vibrios (20, 36). In general, hemagglutination by our isolates was sensitive to inhibition by D-mannose. Analogous results were also reported in *A. hydrophila* (1, 3), *V. anguillarum* (24, 36, 41), and *A. salmonicida* (24) strains. A pattern commonly detected was inhibition by D-mannose and L-fucose. Interestingly, Burke et al. (6) reported that this pattern was more frequent in environmental *Aeromonas* spp. than in clinical isolates. In contrast to Mittal et al. (25), we found that the absence in our isolates of the Acr^- PAB^+ phenotype did not preclude pathogenicity for fish or enterotoxigenicity. Similar results were reported by Janda et al. (15) with mesophilic *Aeromonas* spp. pathogenic for mice. These variable findings can be explained by the heterogeneity showed by the motile *Aeromonas* strains in their antigenic characteristics (10, 21, 28).

Although there are contradictory reports on the possible relationship between cytotoxicity and virulence for fish or enteropathogenicity for humans (7, 26, 36), we demonstrated, using homoiothermic and fish cell lines (Table 2), that cytotoxin production is an adequate criterion of neither enterotoxigenicity nor virulence for fish. In addition, the patterns of cytotoxic activities in our isolates seem to indicate that cytotoxins of motile *Aeromonas* spp. are different from the labile toxin of *E. coli* and more like the *V. cholerae* toxin. A loss of cytotoxic effects was observed after heat treatment (80°C for 15 min) of positive samples, suggesting the involvement of proteolytic enzymes. Biochemical tests such as Voges-Proskauer, production of gas, fermentation of arabinose, and lysine decarboxylase have been associated with enterotoxigenic and cytotoxic capacities of clinical *Aeromonas* strains (6, 8, 17, 43). Nevertheless, few attempts have been made to establish similar relationships in fish isolates (44). Interestingly, we have found that the lysine decarboxylase test seems to be useful for screening enterotoxigenic isolates but not for selecting strains virulent for fish.

All the results of our study indicate that precaution must be taken in the establishment of relationships among pathogenicity for fish or humans, cytotoxin production, and phenotypic properties because the results are influenced by the homoiothermic or poikilothermic systems used in all of the in vivo and in vitro assays.

Up to now, the number and nature of the extracellular toxic substances of *Aeromonas* and *Vibrio* strains have not been clearly determined. Examination of the biological activities of ECP obtained from selected *A. hydrophila* strains pathogenic for fish (Table 3) revealed that they produced a heat-labile substance lethal for fish. The range of lethal doses of these crude ECP (from 18 to 35 μg of protein per g of fish) was comparable to those reported previously for *Aeromonas* and *Vibrio* species (16, 18, 34). In addition, ECP also displayed proteolytic, hemolytic, and cytotoxic activities (Tables 2 and 3) which were lost after heat treatment. Our preliminary data on the purification of the ECP from strain B-32 (T. P. Nieto, Y. Santos, A. E. Toranzo, and A. E. Ellis, Abstr. 3rd Int. Conf. Eur. Ass. Fish Pathol., Bergen, Norway, 1987, p. 37) revealed that proteases rather than hemolysins may be important virulence factors in *A. hydrophila* infections. However, the fact that avirulent strain P-226 showed a strong proteolytic activity indicates, as Thune et al. (35) reported, that both the quantitative and qualitative production of proteases are important in establishing the virulence of a particular strain.

The overall results reported here led us to draw the general conclusion that none of the virulence determinants studied can be considered an absolute criterion of pathogenicity in environmental motile *Aeromonas* spp., since some pathogenic strains lack these virulence properties which, by contrast, are found in nonpathogenic strains. In addition, the fact that both *A. hydrophila* and *A. sobria* strains virulent and nonvirulent for fish were potentially pathogenic with regard to enterotoxigenicity, together with their cytotoxic effects on homoiothermic cells, is a matter of public health concern.

ACKNOWLEDGMENTS

We thank Frank Hetrick, Department of Microbiology, University of Maryland, for kindly supplying fish cell lines and the donors of *Aeromonas* strains. We also thank Javier Barja, Departamento de Algebra, Universidad de Santiago, Santiago de Compostela, for his contribution in the statistical analysis of the data and in the presentation of the results.

This work was supported by grant AQ-018/84 from the Comisión Asesora de Investigación Científica y Técnica, Ministerio de Educación y Ciencia (Spain), and by grant CCB85-09013 from the Comité Conjunto Hispano-Norteamericano para la Cooperación Científica y Tecnológica. Y. Santos acknowledges the Ministerio de Educación y Ciencia (Spain) for a research fellowship.

LITERATURE CITED

1. **Adams, D., H. M. Atkinson, and W. H. Woods.** 1983. *Aeromonas hydrophila* typing scheme based on patterns of agglutination with erythrocytes and yeast cells. J. Clin. Microbiol. **17**:422–427.
2. **Allan, B. J., and R. M. W. Stevenson.** 1981. Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. Can. J. Microbiol. **27**:1114–1122.
3. **Atkinson, H. M., and T. J. Trust.** 1980. Hemagglutination properties and adherence ability of *Aeromonas hydrophila*. Infect. Immun. **27**:938–946.
4. **Boulanger, Y., R. Lallier, and G. Cousineau.** 1977. Isolation of enterotoxigenic *Aeromonas* from fish. Can. J. Microbiol. **23**: 1161–1164.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Burke, K., J. Robinson, M. Cooper, J. Beaman, K. Patridge, D. Peterson, and M. Gracey.** 1984. Biotyping and virulence factors in clinical and environmental *Aeromonas* species. Appl. Environ. Microbiol. **47**:1146–1149.
7. **Cipriano, R. C., B. R. Griffin, and B. C. Lidgerding.** 1981. *Aeromonas salmonicida*: relationship between extracellular growth products and isolate virulence. Can. J. Fish. Aquat. Sci. **38**:1322–1326.
8. **Cumberbatch, N., M. J. Gurwith, C. Langston, R. B. Sack, and J. L. Brunton.** 1979. Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. Infect. Immun. **23**:829–837.
9. **Davis, W. A., J. G. Kane, and V. P. Garagusi.** 1978. *Aeromonas* infections: a review of the literature and a case report of endocarditis. Medicine (Baltimore) **57**:267–277.
10. **Dooley, J. S. G., R. Lallier, D. H. Shaw, and T. J. Trust.** 1985. Electrophoretic and immunochemical analyses of the lipopolysaccharides from various strains of *Aeromonas hydrophila*. J. Bacteriol. **164**:263–269.
11. **Duguid, J. P.** 1959. Fimbriae and adhesive properties in *Klebsiella* strains. J. Gen. Microbiol. **21**:271–286.
12. **Hastings, T. S., and A. E. Ellis.** 1985. Differences in the production of haemolytic and proteolytic activities by various isolates of *Aeromonas salmonicida*, p. 69–77. *In* A. E. Ellis (ed.), Fish and shellfish pathology. Academic Press, Inc. (London), Ltd., London.
13. **Hazen, T. C., C. B. Fliermans, R. P. Hirsch, and G. H. Esch.** 1978. Prevalence and distribution of *Aeromonas hydrophila* in the United States. Appl. Environ. Microbiol. **36**:731–738.
14. **Hsu, T. C., W. B. Waltman, and E. B. Shotts.** 1981. Correlation of extracellular enzymatic activity and biochemical characteristics with regard to virulence of *Aeromonas hydrophila*. Dev. Biol. Stand. **49**:101–111.
15. **Janda, J. M., L. S. Oshiro, S. L. Abbot, and P. S. Duffey.** 1987. Virulence markers of mesophilic aeromonads: association of the autoagglutination phenomenon with mouse pathogenicity and the presence of a peripheral cell-associated layer. Infect. Immun. **55**:3070–3077.
16. **Kanai, K., and H. Wakabayashi.** 1984. Purification and some properties of protease from *Aeromonas hydrophila*. Bull. Jpn. Soc. Sci. Fish. **50**:1367–1374.
17. **Kaper, J. B., H. Lockman, R. R. Colwell, and S. W. Joseph.** 1981. *Aeromonas hydrophila*. Ecology and toxigenicity of isolates from an estuary. J. Appl. Bacteriol. **50**:359–377.
18. **Kodama, H., M. Moustafa, T. Mikami, and H. Izawa.** 1985. Partial purification of extracellular substance of *Vibrio anguillarum* toxigenic for rainbow trout and mouse. Fish Pathol. **20**: 173–179.
19. **Lallier, R., F. Bernard, and G. Lalonde.** 1984. Difference in the extracellular products of two strains of *Aeromonas hydrophila* virulent and weakly virulent for fish. Can. J. Microbiol. **30**:900–904.
20. **Larsen, J. L., and S. Mellergaard.** 1984. Agglutination typing of *Vibrio anguillarum* isolates from diseased fish and from the environment. Appl. Environ. Microbiol. **47**:1261–1265.
21. **Leblanc, D., K. R. Mittal, G. Olivier, and R. Lallier.** 1981. Serogrouping of motile *Aeromonas* species isolated from healthy and moribund fish. Appl. Environ. Microbiol. **42**:56–60.
22. **Lemos, M. L., P. Salinas, A. E. Toranzo, J. L. Barja, and J. H. Crosa.** 1988. Chromosome-mediated iron uptake system in pathogenic strains of *Vibrio anguillarum*. J. Bacteriol. **170**: 1920–1925.
23. **Liu, P. V.** 1957. Survey of hemolysin production among species of pseudomonas. J. Bacteriol. **74**:718–727.
24. **Mellergaard, S., and J. L. Larsen.** 1981. Haemagglutinating activity of *Aeromonas salmonicida* and *Vibrio anguillarum* isolated from diseased rainbow trout (*Salmo gairdneri*). Bull. Eur. Ass. Fish Pathol. **1**:26–28.
25. **Mittal, K. R., G. Lalonde, D. Leblanc, G. Olivier, and R. Lallier.** 1980. *Aeromonas hydrophila* in rainbow trout: relation between virulence and surface characteristics. Can. J. Microbiol. **26**: 1501–1503.
26. **Morgan, D. R., P. C. Johnson, H. L. Dupont, T. K. Satterwhite, and L. V. Wood.** 1985. Lack of correlation between known virulence properties of *Aeromonas hydrophila* and enteropathogenicity for humans. Infect. Immun. **50**:62–65.
27. **Munn, C. B.** 1980. Production and properties of a haemolytic toxin by *Vibrio anguillarum*, p. 69–74. *In* W. Ahne (ed.), Fish diseases. Third COPRAQ-Session. Springer-Verlag KG, Berlin.
28. **Nomura, J., and T. Aoki.** 1985. Morphological analysis of lipopolysaccharide from Gram-negative fish pathogenic bacteria. Fish Pathol. **20**:193–197.
29. **Olivier, G., R. Lallier, and S. Larivière.** 1981. Toxigenic profile of *Aeromonas hydrophila* and *Aeromonas sobria* isolated from fish. Can. J. Microbiol. **26**:330–333.
30. **Reed, L. J., and H. Müench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27**:493–497.
31. **Sakai, D. K.** 1985. Loss of virulence in a protease deficient mutant of *Aeromonas salmonicida*. Infect. Immun. **48**:146–152.
32. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.
33. **Shotts, E. B., T. C. Hsu, and W. D. Waltman.** 1985. Extracellular proteolytic activity of *Aeromonas hydrophila* complex. Fish Pathol. **20**:37–44.
34. **Thune, R. L., T. E. Graham, L. M. Riddle, and R. L. Amborski.** 1982. Extracellular products and endotoxin from *Aeromonas hydrophila*: effects on age-O channel catfish. Trans. Am. Fish. Soc. **111**:749–754.
35. **Thune, R. L., M. C. Johnson, T. E. Graham, and R. L. Amborski.** 1986. *Aeromonas hydrophila* β-haemolysin: purification and examination of its role in virulence in O-group channel catfish, *Ictalurus punctatus* (Rafinesque). J. Fish Dis. **9**:55–61.
36. **Toranzo, A. E., J. L. Barja, R. R. Colwell, F. M. Hetrick, and J. H. Crosa.** 1983. Haemagglutinating, haemolytic and cytotoxic activities of *Vibrio anguillarum* and related vibrios isolated from striped bass on the Atlantic Coast. FEMS Microbiol. Lett. **18**: 257–262.
37. **Toranzo, A. E., J. L. Barja, S. A. Potter, R. R. Colwell, F. M. Hetrick, and J. H. Crosa.** 1983. Molecular factors associated with virulence of marine vibrios isolated from striped bass in Chesapeake Bay. Infect. Immun. **39**:1220–1227.
38. **Toranzo, A. E., A. M. Baya, B. S. Roberson, J. L. Barja, D. J. Grimes, and F. M. Hetrick.** 1987. Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture **61**: 81–97.
39. **Toranzo, A. E., P. Combarro, Y. Conde, and J. L. Barja.** 1985. Bacteria isolated from rainbow trout reared in fresh water in Galicia (Northwestern Spain). Taxonomic analysis and drug resistance patterns, p. 141–152. *In* A. E. Ellis (ed.), Fish and shellfish pathology. Academic Press, Inc. (London), Ltd., London.
40. **Toranzo, A. E., Y. Santos, T. P. Nieto, and J. L. Barja.** 1986. Evaluation of different assay systems for identification of environmental *Aeromonas* strains. Appl. Environ. Microbiol. **51**: 652–656.
41. **Trust, T. J., D. Courtice, A. G. Khouri, J. H. Crosa, and M. H. Schiewe.** 1981. Serum resistance and hemagglutination ability of

marine vibrios pathogenic for fish. Infect. Immun. **34**:702–707.
42. **Trust, T. J., and R. A. H. Sparrow.** 1974. The bacterial flora in the alimentary tract of freshwater salmonid fishes. Can. J. Microbiol. **20**:1219–1228.
43. **Turnbull, P. C. B., J. V. Lee, M. D. Miliotis, S. V. de Valle, H. J. Koornhof, L. Jeffery, and T. N. Bryant.** 1984. Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. J. Clin. Microbiol. **19**:175–180.
44. **Wakabayashi, H., K. Kanai, T. C. Hsu, and S. Egusa.** 1981. Pathogenic activities of *Aeromonas hydrophila* biovar *hydrophila* (Chester) Popoff and Véron, 1976 to fishes. Fish Pathol. **15**:319–325.
45. **West, P. A., and R. R. Colwell.** 1984. Identification of *Vibrionaceae*. An overview, p. 285–263. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.